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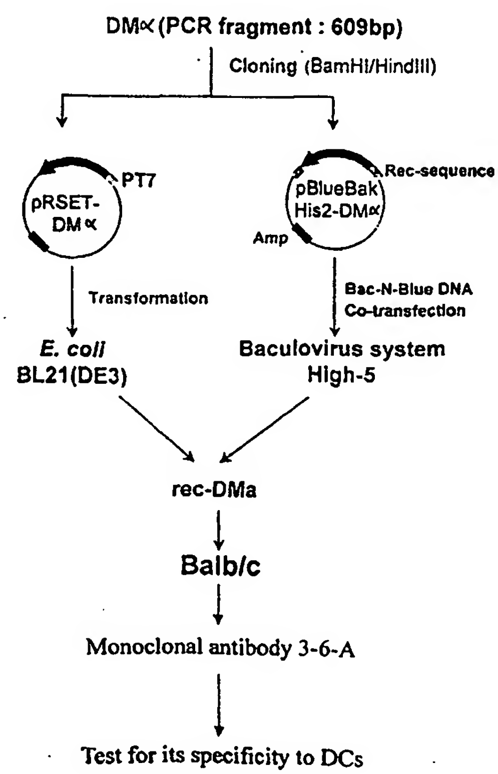
(54) Title: MONOCLONAL ANTIBODY 3–6–A SPECIFIC TO SURFACE OF DENDRITIC CELLS AMONG THE PERIPHERAL BLOOD LEUKOCYTES

(57) Abstract

Disclosed is the monoclonal antibody, 3–6–A, which binds to the extracellular region of a DM α and the hybridoma cell, KHB–DM,with a Deposition No. of KCTC–0485BP, which produces the monoclonal antibody 3–6–A. The monoclonal antibody shows potent binding capacity and exclusive specificity to dendritic cells only, among the peripheral blood leukocytes.



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MONOCLONAL ANTIBODY 3-6-A SPECIFIC TO SURFACE OF DENDRITIC CELLS AMONG THE PERIPHERAL BLOOD LEUKOCYTES.

TECHNICAL FIELD

The present invention relates to a monoclonal antibody 3-6-A that reacts only with dendritic cells. More particularly, the monoclonal antibody 3-6-A reacts with extracellular region of DM α exclusively expressed on the surface of dendritic cells among the peripheral blood leukocytes. Therefore, the present invention relates to the usability of the monoclonal antibody 3-6-A in line with dendritic cell-specific surface marker.

BACKGROUND ART

When being exposed to external antigens as a consequence of pinocytosis or pathogenic infection, dendritic cells (hereinafter referred to as DCs) ingest the antigens, degrade the proteins and present them on their surfaces in the form of peptides bound to MHC class II molecules. Even though the population of DCs are less than 1 % of the total PBL, DCs are much more potent in antigen presentation to T cells than monocytes and macrophages (Pierre *et al.*, *Nature* 388:787 (1987)).

Once sensitized to external antigens, DCs home back to lymph nodes with secreting a specific C-C chemokine. In the lymph nodes, sensitized DCs activate naive T cells (Adema *et al.*, *Nature* 387:713 (1997); Ingulli E *et al.*, *J. Exp. Med.*,

185:2133 (1997)) to induce cellular immunity against the antigens (Steinman R. M., *Annu. Rev. Immunolo.*, 9:271 (1991)). DCs are reported to play an important role in the positive and negative selection of T cells in the thymus (Carlos A., *Immunol. Today* 18). Other experiments have shown that the chemokines secreted from DCs are involved in the homing of T cells to lymph nodes (Adema *et al., Nature* 387:713 (1997); Ingulli *et al., Exp. Med.*, 185:2133 (1997)).

In addition, DCs have a capacity to induce IL-12 and activate cancer cell-specific CTLs which are effective to suppress cancer generation and cancer cell proliferation (Gabrilovich *et al.*, *Cell Immunol.* 170:111-9 (1996): Vezzio *et al.*, *Int. Immunol.* 8:19963-70 (1996); Young *et al., J. Exp. Med.* 183:7-11 (1996)). By taking advantage of these functions of DCs, intensive research has recently been directed to the use of DCs in the development of immuno-therapy for cancer (Gilboa *et al., Cancer Immunol. Immunother.*, 46:82-87 (1998); Nestle *et al., Nat. Med.*, 4:328 (1998); Song *et al., J. Exp. Med.*, 186:1247 (1997)).

Besides, DCs are known to take an essential role in the pathogenesis of AIDS (Fauci, *Science*, 262:1011 (1993); Pantaleo *et al., Nature*, 362:355 (1993); Embretson *et al., nature*, 362:359 (1993); Haynes *et al., Science*, 271:324 (1996)). Patients infected with HIV-1 virus usually undergo asymptomatic period from 3 to 15 years for which only a tracing amount of HIV-1 virus is found in the blood stream of patients. However, a large quantity of the virus and infected CD4+ T cells are found around DCs in lymph nodes (Pantaleo et al., *Nature* 362:355 (1993)). It means that the HIV replication is active in the lymphoid organ throughout the period of clinical latency, even at times when minimal viral activity is demonstrated in blood. For this

phenomenon, they give an explanation as follows: DCs are exposed and infected with a large number of HIV-1 during the primary viremia and return to lymph nodes in which they stimulate naive T cells and then spread the virus to those activated CD4+ T cells. DC-mediated HIV-1 spreading cause active infection of CD4+ T cell and shortage of T cells in lymph nodes, resulting in the depletion of CD4+ T cells in blood stream to the level of AIDS (Blauvelt *et al.*, *Clin. Invest.* 100:2043 (1997); McCloskey *et al.*, *J. Immunol.* 158:1014 (1997)). A report discloses that, when purified, primary CD4+ T cells are not susceptible to HIV-1 in the absence of antigen presenting cells such as DCs or macrophages ,and DCs are much more efficient in transmission of HIV-1 to CD4+ T cells than macrophages (Joo *et al.*, *J. Kor. Soc. Microbiol.*, 30:77 (1995)). These research results are examples that concretely show that DCs are greatly responsible for the AIDS progression.

During the last several years, DCs have been highly focussed by immunologists and related scientists for their special features and characteristics as mentioned above. However, DC studies have not so much progressed as generally expected in other experiments because of the following limitations. First, DC compose less than 1 % of the total PBL and do not increase in their population in vitro even in the presence of granulocyte macrophage-colony stimulating factor (GM-CSF) which has been reported to support the DC survival up to 6 weeks (Marcowitz and Engleman, J. Clin. Invest. 85:955, 1990). Second, human DC-specific surface markers have not yet been identified.

Limited cell population and lack of a DC-specific surface marker have made it difficult to use pure and enough amounts of DCs for further experiments.

Nonetheless, several laboratories persisted with their investigations, leading to the current acceptances about the special characteristics of DCs. Up to now, DCs have been purified from the PBL generally through the negative selection procedure described elsewhere (Freudenthal *et al., Proc. Natl. Acad. Sci* 87:7698 (1990)). In the negative selection method, DCs are isolated by removing other immunocytes such as T cells, B cells, monocytes and macrophages from the PBL through the following experimental procedures; Ficoll gradient, E-rosetting, adhesive panning, Fc panning, metrizamide gradient centrifugation, and antibody panning processes. These procedures are so pains-taking and sophisticate that the procedures may not be applicable to general laboratories.

Recently, several investigators have tried to generate DCs by differentiating bone marrow stem cells (CD34+, CD11c+) or monocytes *in vitro* in the presence cytokines such as GM-CSF and IL-4 (Bender et al., J. Immunol. Methods 196:121-135 (1996)). Most of the recent DC studies for immunotherapy exploit this method to prepare DCs. However, this method also has its own disadvantages in that it requires a high cost and takes a long period of time for differentiation. In addition, even though the differentiated cells look like DC morphology, these cells are still in controversy for their biological functions whether they are authentic DCs and are able to take place real DC *in vivo* when required in the immunotherapy.

Therefore, in order to study DCs to a practical level, first of all, it is very essential to identify DC-specific surface markers and generate monoclonal antibodies against it. Those monoclonal antibodies would be very useful not only to study DCs but also applicable to positive selection of DCs from the PBL.

For that purpose, a few monoclonal antibodies have been suggested and used for DC-detecting antibody. However, most of them are not suitable enough to be used for detection and isolation of DCs from PBL owing to their own limitations. For instance, monoclonal antibody (mAb) against HB15 molecule (CD83) reacts only with activated DCs, but not with the naive DC at all. Our present investigations have shown that CD83 is not sufficiently expressed even in the activated DCs when compared to other cell-specific surface markers. Moreover, the mAb against CD83 is not so much specific as it reacts with activated monocytes /macrophages as well as with activated DCs. Nevertheless, It has been widely used in identifying the monocyte-derived DCs *in vitro* (Fearnley *et al., Blood*, 89:3708 (1997); Zhou *et al., J. Immunol.*, 154:3821 (1995)).

Besides, CD11c and CD1a have been reported as DC-specific surface markers (Gao *et al., Immunology* 91:135 (1997); Lardon *et al., Immunology* 91:553 (1997); Ruedl *et al., Immunol.* 266:1801 (1996)). However, they have a problem in their specificity to DCs. CD11c is expressed not only in DCs but also in macrophages, granulocytes and NK cells, while CD1a is found on the thymocytes and Langerhans cells, as well. These results are partially confirmed in our present experiments.We found that CD11c was expressed substantially on the surface of DCs, but was also expressed on the B cells and monocytes in a similar or reduced amount. Whereas, CD1a was not expressed on the surface of naive DCs. These results indicate that CD1a, CD11c and CD83 are not suitable for DC-specific surface markers.

DISCLOSURE OF THE INVENTION

With this background in mind, the present inventors made a cDNA library of DC and examine the library to seek for a DC specific surface marker. The inventors found that the HLA-DM α/b (hereinafter referred to as DM α) genes are expressed only in DCs among the PBLs through a series of experiments, plaque-lifting and southern blot differential hybridization, sequencing and quantitative RT-PCR (Bae *et al., Mol. Cells,* 5:569, 1995). A monoclonal antibody was generated against extracellular region of DM α, and then called 3-6-A. Mab 3-6-A was tested for its specificity to DCs together with other suggested monoclonal antibodies.

Therefore, it is an object of the present invention to provide a novel monoclonal antibody, which exclusively recognizes DCs among the PBL.

It is another object of the present invention to provide a hybridoma cell, which produces the novel monoclonal antibody.

In order to produce a monoclonal antibody against the DM α protein, which is specifically expressed in DCs among PBLs, there was made an attempt in which the DM α protein was expressed in E. coli and inoculated into BALB/c mice for immunization. Any of the monoclonal antibodies thus obtained, however, were revealed to be incapable of recognizing normal DM α which is expressed in DCs or Raji cells (Kim *et al., Mol. Cells*, 6:684 (1996)). DM α shows amino acid sequence homology over 75% in between human and mouse, suggesting that it would be

difficult to obtain any anti-human DM α monoclonal antibody in BALB/c mice with recombinant h-DM α protein.

The reason why the monoclonal antibodies generated by Kim et al (Mol, Cells, 6:684 (1996)) could not recognize the normal DM α expressed in DCs, is believed to be attributed to the following possibilities. The recombinant DM α would be lost with its B-cell epitopes by lacking in glycosylation or surructural disruption when expressed as a form of inclusion body.

In the present invention, recombinant DM α proteins were obtained from a baculovirus system. After being proven to maintain the antigenicity of the normal DM α expressed in Raji cells or DCs, the recombinant DM α proteins were used to generate the monoclonal antibody 3-6-A. The monoclonal antibody 3-6-A of the present invention was found to be an IgG1 subclass having γ1 isotype κ-chain as analyzed by IsoStrip TM kit (Boehringer Mannheim).

The monoclonal antibody 3-6-A shows a strong reactivity to the normal DM α expressed in DCs and Raji cells, and the reactivity is very specific only to DCs among the primary immunocytes. In addition, the antibody 3-6-A was found to stain deeply not only the cytoplasm but also the cell surface of DCs. These properties indicate that only DCs among the PBL express DM α in a substantial amount on their surface as well as in the cytoplasm. This result is quite different from the previous reports addressing that the DM proteins are endosomal compartments and are localized only in the cytoplasm (Karlsson et al., Science, 266:1569 (1994); Sanderson et al., Science, 266:1566 (1994)). Present inventors found that the

discrepancy was attributed to the different cell types used for the experiments. Actually, Raji cells were stained with 3-6-A only when fixed and permeabilized first, but not stained without fixation, indicating that DM is localized only in the cytoplasm in Raji cells, but not on the cell surface, as reported previously. These results strongly suggest that the DM expression pattern is likely to be cell type specific. Present inventors found first in the world that the DM is exclusively expressed on the surface of DCs among the PBL.

Recently, several reports have addressed that DM play an important role in MHC class II-dependent antigen presentation as a sharperon protein (Morris et al., Nature 368:551, 1994 ; Karlsson et al., Science 266:1569, 1994). Sanderson et al (Immunology 4:87, 1996) reported by co-immunoprecipitation experiments that DM induces CLIP dissociation from the HLA-DR and facilitates peptide binding to DR molecules. But the present finding that substantial amounts of DM are expressed on the surface of DC suggests that the DM molecules have some other important funtions in DC, at least in parts, as well as a chaperon function. First of all, our findings strongly suggest that the DM molecules can be used as DC-specific surface markers.

The monoclonal antibody 3-6-A of the present invention can be used not only for the investigation of the DM functions when expressed on the surface of DCs, but also for the development of devices used for the positive selection of DCs among the PBLs. DCs play an essential role in immune responses. Particularly, DCs are essential materials to study the immunotherapy against cancer. Accordingly, it is urgently demanded to develop a positive selection method for the purification of

DCs. Once established by using the monoclonal antibody 3-6-A, developed in the present invention, the positive selection device will be very helpful to study DC-mediated researches and the immunotherapy against cancer. The present invention was accomplished by preparing the monoclonal antibody 3-6-A on the basis of the research results of the inventors and assaying it for its specificity and binding capacity to DCs.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a flow diagram briefly showing the procedures used for generation and characterization of the monoclonal antibody 3-6-A;

Fig. 2 is a SDS-PAGE photograph showing the expresion of recombinant DM α a in a transformed E. coli;

Fig. 3 is a photograph showing positive plaques formed as a consequence of the production of recombinant virions in the High-5 cells when transfected with a recombinant baculovirus DNA;

Fig. 4 is a Western blot photograph showing the normal expression of a recombinant DM α in the baculovirus system;

Fig. 5 is a Western blot photograph showing the reactivity of mouse anti-recombinant DM α antiserum to the authentic DM α expressed in Raji cells;

Fig. 6 is a Western blot photograph showing the reactivity of the monoclonal antibody 3-6-A to the DM α expressed in E. coli and cells;

Fig. 7A is a fluorescence microscopic photograph showing the the cytoplasm and surface of DCs and Raji cells stained with the monoclonal antibody 3-6-A;

Fig. 7B is a FACS histogram of the Raji cells when stained with the monoclonal antibody 3-6-A before or after fixation;

Fig. 8 is a fluorescence microscopic photographs showing the specificity of the monoclonal antibody 3-6-A to DCs in comparison with those of other commercially available DC-specific monoclonal antibodies when used forstaining the purified primary immune cells;

Fig. 9 is a dot immunoblot photograph comparing the reactivity and specificity of the monoclonal antibody 3-6-A and commercially available, DC-specific monoclonal antibodies to purified DCs; and

Fig. 10 is a FACS histogram showing the reactivity of the monoclonal antibody 3-6-A to purified primary immune cells, respectively.

BEST MODES FOR CARRYING OUT THE INVENTION

To accomplish the objects of the present invention, first, a 609 bp DNA fragment coding for an extracellular region (corresponding to a base sequence from 122-731) of a DMα gene is cloned into a baculovirus transfer vector, pBlueBacHis2A, to give a recombinant plasmid, pBlueBacHis2A-DMα. This recombinant plasmid, together with a baculovirus full-length DNA, Bac-N-Blue (Invitrogen), is co-transfected into High-5 cells (Invitrogen), resulting in the production of a recombinant baculovirus Bac-N-Blue-DMα, which is then infected

into mass cultured High-5 cells. The infected High-5 cells are harvested, whose extracts are applied to the Ni^{+}-NTA column (Qiagen) for purification of the recombinant DM α protein. BALB/c mice are immunized with the pure recombinant DM α protein and their splenocytes are excised and undergo hybridization with mouse myeloma Sp2/0 cells, resulting in production of the hybridoma cell, KHB-DM, which produce the monoclonal antibody 3-6-A. The monoclonal antibody 3-6-A is now being obtained by culturing the hybridoma cell, KHB-DM.

EXAMPLE 1: Purification of DC and Other Primary Immune Cells.

Either from a buffy coat or leukopak, supplied from Korean Red Cross Blood Bank, DCs and other immune cells (T cells, B cells and monocytes) were purified through the procedures as reported previously (Bae et al., Mol. Cells, 5, 569, 1995).

EXAMPLE 2: mRNA Extraction and DM α -cDNA Synthesis from the DCs

From the DCs obtained in Example 1, mRNA was extracted according to a known technique (Bae et al., Mol. Cells, 5, 569, 1995). The mRNA was incubated with 1 μg of an oligo-dT primer at 37 °C for 2 min in a reverse transcription solution (RNase inhibitor 0.5 μl, 5x first strand synthesis reaction buffer 4 μl, 0.1 mM DTT 2 μl, 10 mM dNTP 1 μl), followed by the addition of 5 units of an AMV-reverse transcriptase. This solution was incubate at 37 °C for 40 min and

then further incubated at 45 ° C for 30 min to synthesize cDNA. A DM α gene was amplified by PCR using the cDNA as template and primers shown below at Table 1.

TABLE 1 : Primers for DM α

BamH I-DM Sense primer	5'-GAT AAG GAT CCG TCC AAG CTC CTA-3'	122-137
HindIII-DM Anti-sense primer	5'-GT TCA AAG CTT TCA CTC CAG CAG ATC TGA GGG-3'	732-712

The PCR was performed by repeating a thermal cycle consisting of 94 ° C/1 min, 40 ° C/30 sec and 72 ° C/45 sec, 5 times, and then, a thermal cycle consisting of 94 ° C/1 min and 72 ° C/1 min, 25 times, so as to produce a 609 bp DNA fragment encoding an extracellular region (base sequence 12-732) of the DM α gene.

EXAMPLE 3: Cloning and Expression of DM α in E. coli

The DNA fragment amplified by PCR in Example 2 was double digested with BamH I and Hind III and inserted into the plasmid pRSET/A (Invitrogen) opened with the same restriction enzymes, to give a recombinant plasmid pRSET-DM α. This recombinant pRSET-DM α was transformed into E. coli BL21(DE3) which was, then, spread on an LB medium containing ampicillin. The positive colonies were inoculated in a M-9 ampicillin broths (1% bactotryptone, Na_2HPO_4, KH_2PO_4,

NaCl, NH_4Cl, 2M glucose, 0.5% diamine, 1M $MgSO_4$, 1M $CaCl_2$) and cultured with agitation. When the absorbance of the cultures reached 0.5, IPTG was added to a final concentration of 1 mM to induce protein expression at 37 ° C for 2 hours. The expression of recombinant DM α in the transformed E. coli was identified by SDS-PAGE analysis in which a band was detected at 29 kDa (Fig. 2). In Fig. 2, lane 1 is a protein size marker, lane 1 the lysate of E. coli BL21 (DE3) transformed only with the pRSET/A plasmid, lane 2 the lysate of E. coli BL21 (DE3) transformed with the recombinant DM α plasmid and lane 3 a DM α protein sample purified from the recombinant bacteria of lane 2 with the aid of an Ni^+-NTA resin (Qiagen).

EXAMPLE 4: Construction of Recombinant Baculovirus Expressing DM α

The DNA fragment amplified by PCR in Example 2 was digested with BamH I and Hind III and inserted into the plasmid pBlueBacHis2A (Invitrogen) opened with the same restriction enzymes. The ligate was transformed into E. coli BL21 (DE3) which was then cultured in the same manner as described in Example 2 to obtain a recombinant plasmid pBlueBacHis2A-DM α. Together with the baculovirus full-length DNA Bac-N-Blue (Invitrogen) linearized by Bsu36I digestion, the recombinant plasmid pBlueBacHis2A-DM α was transfected into High-5 cells (Invitrogen) with the aid of Lifofectin (Gibco BRL). Five to 7 days culture in a liquid medium after transfection, the supernatant was harvested and reinfected at a diluted concentration into High-5 cells. Two days after inoculation, the cells were covered

with a 1% low-melting agarose mixture (prepared by mixing 50 mg/ml of X-gal, 2x TNM-FH medium with 2% low-melting agarose in an equal volume) and cultured for 3-5 days further. Blue plaques were selected as positive clones (panel A in Fig. 3). The selected clones were further purified by repeating the above culturing procedures three times. They were amplified in High-5 cells to obtain a large quantity of recombinant baculovirus Bac-N-Blue-DM α.

EXAMPLE 5: Analysis of the Cloned DM α in the Recombinant Baculovirus

From the recombinant baculovirus Bac-N-Blue-DM α obtained in Example 4, the genomic DNA was extracted for PCR analysis. PCR was performed using the primers Bac1/Bac2 of Table 2 (included in the Bac-N-Blue transfection kit, Invitrogen) with the genomic DNA serving as a template to identify the recombinant baculovirus is harboring the DM gene.

TABLE 2: Bac1/Bac2 primers

Bac 1 primer	5'-TTT ACT GTT TTC GTA ACA GTT TTG	-44~ -21
Bac 2 primer	5'-CAA CAA CGC ACA GAA TCT AGE-3'	+794~ +77

EXAMPLE 6: Expression and purification of DM α from the Recombinant Baculovirus-infected High-5 cells.

High-5 cells were infected with the recombinant baculovirus Bac-N-Blue-DMα at 5 MOI and then cultured at 27 °C for 5 days in a humid incubator. The recombinant DMα proteins were extracted from the infected cells and purified by using the Ni^+-NTA resin column (Qiagen).

EXAMPLE 7: Immunization of BALB/c mouse with Recombinant DMα

The DMα protein obtained in Example 6 was mixed with an equal volume of the Freund's adjuvant (Sigma) and inoculated into 5-6 week old BALB/c mice at a dose of 50 μg/mouse. Inoculated mice were then boosted secondarily and tertiarily with the same antigen at a dose of 25 μg/mouse in the 3 weeks interva. After the tertiary immunization, Each BALB/c mouse was bleed from the ocular vein to analyze the antiserum.

EXAMPLE 8: Reactivity of Antiserum to the Recombinant DMα

The High-5 cells infected with the recombinant baculovirus Bac-N-Blue-DMα were homogenized and the proteins obtained from the disrupted cells were separated by SDS-PAGE. The proteins separated on an SDS-PAGE were transblotted to a nitrocellulose membrane using a transferring solution (48 mM tris-HCl, 39 mM glycin, 20% methanol, 1.3 mM SDS) in a semi-dry gel blotter (Bio-Rad). This blotted nitrocellulose membrane was blocked with a blotto (5% (w/v)

non-fat milk, 0.02% NaN_3 in PBS) for 30 min, washed three times with PBS, and placed for 30 min at room temperature in a 10 ml PBS solution containing 5 μl of the antiserum obtained in Example 7. Washing 3 times with PBS, membrane was soaked in the PBS solution containing goat anti-mouse-IgG-alkaline phosphatase (Sigma) as a second antibody, and then visualized by adding a NBT/BCIP solution {66 μl of 50 mg/ml NBT (nitro blue tretrzolium) and 30 μl of 50 mg/ml BCIP (5-bromo-4-chloro-3-indolyl phosphate) in 10 ml of an AP buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.5)} as a substrate for the enzymereaction. When a band appeared as a consequence of the enzymatic reaction in a dark place, stop solution (20mM EDTA, 150mM NaCl in 10 mM Tris-Cl, pH 8.0) was added to terminate the reaction.

This Western blotting analysis showed that the recombinant baculovirus Bac-N-Blue-DMα normally expressed DMα in High-5 cells, as shown in Fig. 4. In Fig. 4, lane M is a protein size marker, lane 1 an uninfected High-5 cell lysate, lane 2 a wild baculovirus-infected High-5 cells lysate, lane 3 lysate of High-5 cell infected with the recombinant baculovirus bac-N-Blue-DMα, lane 4 E. coli BL21(DE3)lysate as a negative control, and lane 5 lysate of the recombinant E. coli BL21(DE3) expressing the recombinant DMα.

EXAMPLE 9: Reactivity of Antiserum to Authentic DMα

Western blot analysis was carried out in a similar manner to that of Example 8, using $1x10^6$ untreated Raji cells and $1x10^6$ Raji cells treated with γ-interferon (Sigma) at a concentration of 100 unit/ml for 3 days. The result is given in Fig. 5. In Fig. 5, lane 1 is a control Jurkat cell lysate, lane 2 a Raji cell lysate and lane 3 the lysate of the Raji cells treated with γ-interferon.

This analysis shows that the antiserum obtained from the BALB/c mice immunized with the recombinant DMα reacts well with the authentic DMα expressed in Raji cells.

EXAMPLE 10: Production of Hybridoma cells Expressing Monoclonal antibody

The mice immunized with the recombinant DMα, obtained from the expression in a baculovirus system in Example 7, were euthanized by backbone dislocation and their spleens were excised. $1.4x10^7$ spleenocytes were suspended in 10 ml of a glucose-rich DMEM medium, mixed with $3x10^6$ cells of the mouse myeloma cell SP2/O (Sp2/O-Ag14 KTCC CRL1581) and washed with a glucose-rich DMEM medium. To the cell pellet, 1 ml of a 50% PEG-4000 solution (Gibco BRL) was added for 1 min to induce cell fusion. After a glucose-rich DMEM medium was slowly added at a rate of 1 ml/min, the cells were well mixed and centrifuged. The cell pellet was suspended in 5 ml of 1x $DMEM_{20\%}$ and distributed to the multiwell plate at an aliquot of 50 μl per well where mouse macrophages were cultured as a feeder cell (Antibody Lab., Mannual ed. H. D. Lane, p220, 1989) and then cultured for one day. Then, an equal volume of 2x HAT medium was added

to each well. During a long culture period of time, fused cells were colonized. Hybridoma cells colonized in HAT medium were selected from 18 wells.

EXAMPLE 11: Identification of Hybridoma Cells Secreting Monoclonal Antibody Reacting with DM α

Each of the 18 colonies obtained in Example 10 was cultured in a large volume of a medium and the supernatant was analyzed by ELISA, dot immunoblot hybridization and Western blot hybridization. 96-well plates were coated overnight at 4 °C with the DM α proteins prepared in Examples 3 and 6 to a final concentration of 0.5 μg per well. Plates were washed twice with 100 μl of PBS containing 0.05 % Tween 20. A 1% non-fat milk solution (dissolved in deionized water, added with 0.02% NaN_3) was added to each wells at an amount of 90 μl per well and allowed to stand at 37 °C for 1 hour. Subsequently, reaction was performed with the antiserum of Example 7 for 37 °C for 1 hour in the wells. After washing, a secondary antibody (goat-anti-mouse-IgG-AP 1/1000 diluted) was added and incubated at 37 °C for 2 hours. After being washed twice, each wells were filled with an alkaline phosphatase substrate solution (p-nitrophenyl phosphate 1 mg in 10% diethanol amine buffer 1 ml (diethanol amine 97 ml, NaN_3 0.2 g (0.02%), $MgCl_2$ $6H_2O$ 100 mg (0.01%) in DDW 800 ml; total 1 liter)) 50 μl per well and incubated at 37 °C for 30 min.

When the reaction was completed, absorbance was measured with an ELISA reader (Molecular Devices) at 405 nm. Western blotting was carried out in the same manner as in Example 8, while dot immunoblot hybridization was performed following the previous method (Kim et al., Mol. Cells, 6,684, 1996).

The results are summarized at Table 3, below. At Table 3, the reactivity of the monoclonal antibody to the recombinant DMα protein expressed in E. coli and baculovirus system, was measured by ELISA and Western blot hybridization while the reactivity to authentic DMα expressed in various cells were measured by dot immunoblot and Western blot hybridization. The experiment have shown that the 14 monoclonal antibodies all strongly react with the recombinant DMα expressed in E. coli, but none of them, except the monoclonal antibody 3-6-A, reacts with the recombinant DMα expressed in High-5 cells. The monoclonal antibody 3-6-A was found to have strong reactivity to DCs among the PBLs as measured by Western blot and dot immunoblot hybridization. Also, the monoclonal antibody 3-6-A was highly reactive to Raji cells in Western blot hybridization. A weak positive response was occasionally detected in B cell fractions in Western blot hybridization. This weak detection offers the possibility that activated B cells might express DM. Based on these results, the monoclonal antibody 3-6-A against DMα was selected out of the 14 monoclonal antibodies. The hybridoma cells which express the monoclonal antibody 3-6-A, were named KHB-DM and were deposited in the Korean Collection for Type Cultures, Korean Research Institute of Bioscience and Biotechnology (deposition No. KCTC-0485BP).

EXAMPLE 12: Reactivity of Monoclonal Antibody 3-6-A to the Authentic DM α Expressed in DCs and Raji Cells

Monoclonal antibody 3-6-A was tested for its reactivity to the recombinant DM α and authentic DM α normally expressed in Raji cells and DCs in Western blot hybridization. The result is given in Fig. 6. As shown in Fig. 6, the monoclonal antibody 3-6-A is highly reactive to the 34 kDa DM α, expressed normally in Raji and DCs. In Fig. 6, lane 1 is a negative control of E.coli BL21 (DE3) lysate, lane 2 a lysate of E. coli expressing DM α, lane 3 a lysate of High-5 cells expressing DM α, lane 3 a Raji cell lysate, and lane 5 a DC lysate.

TABLE 3: Binding Capacity of Monoclonal Ab to DM α

Abs	Antigens							
	Recombinant DM		Cells					
	E. coli	High-5	PBL	T	B	MC	DC	Raji
DM α pAb	++++	++++	+	-	±	-	++	++
1-1-H	++	-	±	-	-	-	-	-
1-3-A	++++	-	-	-	-	-	-	-
1-8-G	++++	-	-	-	-	-	-	-
1-10-A	+++	+	-	-	-	-	-	-
1-11-E	++++	-	-	-	-	-	-	-
2-11-G	++++	-	-	-	-	-	-	-
2-12-A	++++	-	-	-	-	-	-	-
3-4-B	+++	-	-	-	-	-	-	-
3-5-B	++++	-	-	-	-	-	-	-
3-6-A	++++	+++	+	-	±	-	+++	+++
4-1-C	++	-	-	-	-	-	-	-
4-2-B	++++	-	-	-	-	-	-	-
5-3-G	+++	-	-	-	-	-	-	-
5-5-D	++++	-	-	-	-	-	-	-

note: biding capacity was measured by ELISA, dot immunoblot and/or Western blot hybridization.

- : results similar to those of negative controls

+ : positive results, the number of this symbol shows the intensity of the positive signal, and is increased one by one whenever the absorbance at 405 nm increases by 0.2 from the basic positive value which is the mean value of negative control plus 5SD (standard deviation). ++++ is used for all reactions whose ELISA value is equal to or higher than 4 symbols.

In Western blot and dot immunoblot, the symbol '+' was used only when a detectable signal is clearly repeated. The number of symbols is increased as the comparative intensity increases.

± : weak or non-repetitive signal.

EXAMPLE 13: Mass Production of Monoclonal Antibody 3-6-A

The hybridoma cell KHB-DM was seeded at a density of $2x10^5$ cells per ml of a high glucose $DMEM_{20\%}$ media. Four days later at a maximum growth, culture supernatants were harvested as a monoclonal antibody 3-6-A source. At this time, the concentration of monoclonal antibody reached to approximately 40 μg per ml of the culture medium.

In order to produce a large amount of the monoclonal antibody, hybridoma cells were cultured in mouse abdominal cavity. BALB/c mouse was injected first

with 1 ml of prestane intraperitoneally. One week later, the mice were inoculated with $5x10^6$ hybridoma cells per mouse. After 7-14 days, when the abdominal cavities of the mice were swollen enough, ascitic fluid was taken by 5-10 ml per mouse with 18-gauge needle attached to a 10 ml syringe, and then spin at 1,500 rpm for 10 min. The supernatant obtained was added with 0.02 % NaN_3 and stored at -70 ° C or less. The monoclonal antibody concentration was 5-9 mg per ml in the ascitic fluid.

EXAMPLE 14: Isotyping of Monoclonal Antibody 3-6-A for H and L chains

The monoclonal antibody 3-6-A produced by the hybridoma cell KHB-DM in Example 11 was examined for its isotype using the isotyping kit, IsoStrip™ (Boehringer mannheim). The monoclonal antibody 3-6-A was found IgG1 isotype having κ-light chain.

EXAMPLE 15: Monoclonal Antibody 3-6-A Staining the Cytoplasm and Surface of DCs

Microscopic slide glass were coated for 15 min with a 1 mg/ml poly-L-lysine (Mw 400,000) solution and washed with deionized distilled water. Raji cells (ATCC CCL86) and DCs, isolated as in Example 1, were diluted to 10^5 cells/ml, attached to the slide coated with poly-L-lysine and then washed with PBS. 100 μl of the monoclonal antibody (culture supernatant) obtained in Example 13 were added to the

cell-attached slide, which was allowed to stand for 1 hour in a CO_2 incubator and then washed three times with PBS. After reaction with the second antibody (anti-mouse-IgG-FITC) at 37 ° C CO_2 incubator for 30 min, the slide was washed twice with PBS and then observed under a fluorescence microscope (Nikon E-600 Epi-fluorescent microscope).

To stain the cytoplasm of DC with 3-6-A, the DCs-attached slides were fixed by immersing them in an organic solvent (50% methanol and 50% acetone) prior to staining with the monoclonal antibody 3-6-A. After fixation, DCs were stained with the monoclonal antibody 3-6-A and the second antibody in the same manner as above and observed in the fluorescence microscope.

In Fig. 7A, panel a is a microscopic photograph of DC, magnified by 400 times at a microscope (Nikon TMS), panel b is a fluorescence microscopic photograph of a DC stained with the PE-conjugated HLA-DR (Becton-Dikinson) as a control, panel c is that of a DC bound and stained with the antibody 3-6-A and FITC-conjugated second antibody,respectively, panel d is that of a DC which were fixed first and then stained in the same manner shown in panel c, panel e and f are those of unfixed and fixed Raji cells, respectively, which were stained in the same manner.

Fig. 7B shows the FACS results of unfixed and fixed Raji cells stained with the monoclonal antibody 3-6-A and FITC-conjugated second antibody. Half of a Raji cell fraction was fixed with Cytofix/cytoperm Cytostain™ Kits(Pharmingen) by following th evender's manual. Unfixed and fixed Raji cells were treated with the monoclonal antibody 3-6-A at 4 ° C for 30 min, washed twice with PBS, and then stained at 4 ° C for 30 min with the same FITC-conjugated second antibody as

mentioned above. Stained cells were washed twice with PBS and analyzed immediately by FACStar (Becton-Dickinson). In Fig. 7B, panel a and b are the FACS results of unfixed and fixed Raji cells, respectively.

As apparently shown in Fig. 7, DM α is expressed in a large quantity on the surface of DC as well as in its cytoplasm (Fig. 7A panel c and d), which is contrary to the previous reports insisting that DM is only an endosomal compartment. Whereas, Raji cells were also deeply stained by the 3-6-A when fixed prior to staining (Fig.7A panel f and 7B panel b). Unfixed Raji cells, however, were not stained at all with the same antibody (Fig.7A panel e and 7B panel a). The results from Raji cells indicate that the DM molecules are localized only in the cytoplasm, but not on the surface of Raji cells, as mentioned in the previous reports (Karlsson et al., Science 266; Science 226:1599-1573, 1994; Sanderson et al., Science 266:1566-1569, 1994).

EXAMPLE 16: Specificity of Monoclonal Antibody 3-6-A to DC Surface

T cells, B cells, monocytes and DCs were isolated in the same manner as in Example 1. Each cells were diluted to 10^5 cells per ml of PBS and treated at 4 °C for 30 min with 100 μl of the monoclonal antibody 3-6-A (culture supernatant) obtained in Example 13. After centrifugation at 750 g, the cell pellet was washed twice with PBS, suspended in 200 μl of PBS and stained at 4 °C for 30 min with 10 μl (1 μg) of the second antibody, anti-mouse-IgG-FITC (sigma). After centrifugation, the cell pellet was washed twice with PBS and attached to a coating slide. Stained cells

were analyzed with a fluorescence microscope as mentioned in Example 15 and its results are given in Fig. 8. In Fig. 8, column T stands for T cells, column B for B cells, column Mc for monocytes/macrophages, and column DC for DCs. Samples were reacted with commercially available various monoclonal antibodies and the monoclonal antibody 3-6-A and then stained with an FITC-conjugated second antibody.

As shown in the bottom of Fig. 8, the monoclonal antibody 3-6-A highly reactive with DCs, but not with other immune cells at all. These data demonstrate that DM molecules are exclusively expressed on the surface of DCs among primary immune cells. In contrast, the antibodies against CD11c or CD83, widely used for DC analysis, are not so much specific to DCs among the PBL as shown in Fig 8. They react with not only DCs, but also B cells and monocytes. Particularly, as for CD83, known to be expressed in the activated DC fraction (Zhou L. J., J. Immunol. 149, 735, 1992), its monoclonal antibody (anti-CD83: Immunotech Co.) showed reactivity, even if weak, to monocytes as well as to naive DCs in a similar intensity. Monoclonal antibody to Fascin, reported allegedly to be expressed only in the cytoplasm of DCs, could not recognize the DCs when unfixed. From these data apparently shown in Fig. 8, it was reconfirmed that the monoclonal antibody 3-6-A produced from the present invension has higher specificity and more potent affinity to DCs than any other conventional monoclonal antibodies.

EXAMPLE 17 Affinity of The Monoclonal 3-6-A to DCs

The affinity of the monoclonal antibody 3-6-A to DCs was compared with those of other conventional DC-specific monoclonal antibodies. DCs were isolated in the same manner as in Example 1 and subjected to dot immunoblot hybridization (Kim *et al.*, *Mol. Cells*, 6, 684, 1996) with the monoclonal activity 3-6-A, together with other commercially available monoclonal antibodies.

Each fraction of DCs containing 10^5 cells, isolated in the same manner as in Example 1, were stained with 1 μg of each of an anti-CD1a monoclonal antibody (Becton-Dickinson), an anti-CD11c monoclonal antibody (Becton-Dickinson), an anti-CD83 monoclonal antibody (Immunotech. Co.), an anti-Fascin monoclonal antibody (granted from NIH AIDS Research and Reference Reagent Program), and the monoclonal antibody 3-6-A in the same manner as in Example 15. Cells treated with the first antibodies were stained with an alkaline phosphatase-conjugated with goat-anti-mouse-IgG (Promega) in the same manner as in Example 15, followed by being blotted to a nitrocellulose membrane with the aid of a dot blotter (Bio-Rad). The membrane was subjected to the chlomogenic reaction as shown in Example 8.

The dot immunoblot hybridization result is given in Fig. 9. In Fig. 9, the dot blot signal of 1, 2, 3 and 4 result from the experiments of DC staining with anti-CD1a, anti-CD11c, anti-CD83 and the anti-Fascin monoclonal antibodies, respectively as controls. Whereas, the dot blot signal 5 shows the DC fraction stained with the monoclonal antibody 3-6-A. Consistent with the result shown in Example 16, the monoclonal antibody 3-6-A shows the most potent affinity to DCs among the monoclonal antibodies tested (Blot 5 in Fig. 9). Next to the monoclonal antibody 3-6-A, the anti-CD11c antibody was the most potent in the affinity to DCs

(Blot 2 in Fig. 9), but was also reactive with monocytes and B-cell fraction (Fig. 8). The antibodies against CD83 or Fascin were found inefficient to react with naïve DCs.

EXAMPLE 18: FACS Analysis of Primary Immune Cells with Monoclonal Antibody 3-6-A

The primary immune cells, including T cells, B cells, monocytes and DCs, isolated as in Example 1, were treated with the monoclonal antibody 3-6-A at 4 ° C for 30 min. Being washed twice with PBS, cells were treated with the second antibody goat-anti-mouse-IgG-FITC (Sigma) at 4 ° C for 30 min. Stained cells were washed twice with PBS, and then immediately analyzed with the FACStar plus (Becton-Dickinson).

The FACS analysis results are given in Fig. 10. In Fig. 10, panel A shows FACS diagrams of the immune cells puried in the same manner as in Example 1, while panel B shows FACS diagrams of the immune cells purified from the PBL cultured for 1 week in an $RPMI_{10\%}$ medium prior to isolation. The FACS diagrams for T cells are in column 1, for monocytes in column 2, for B cells in column 3 and for DCs in column 4. As shown in columns 1 and 2, neither T cells nor monocytes /macrophages, whether isolated primarily or from the cultured PBL, reacted with the monoclonal antibody 3-6-A. As for B cells, when primarily isolated, they did not react with the monoclonal antibody 3-6-A at all. In contrast, when isolated from the PBL cultured for one week, tiny amounts of B cell fraction were stained with the

antibody 3-6-A (column 3 in Fig. 10). Whereas, more than 60% of the isolated DC fractions were deeply stained with the monoclonal antibody 3-6-A. Not a big difference was found in the FACS results between the cells isolated primarily and after the culture for one week (column 4 in Fig. 10). These FACS data also demonstrate that the monoclonal antibody 3-6-A reacts specifically with DCs among primary immunocytes.

EXAMPLE 19: Examination of the Antigenicity between DM α Proteins Expressed in E. coli and Baculovirus System Using Antiserum

The same procedure as in Example 7 was repeated to obtain antisera (anti-DM α -polyclonal antibodies) from the mice immunized with the recombinant DM α proteins which were produced from E. coli in Example 3 and from the baculovirus system in Example 6. Using the polyclonal antibodies, the recombinant DM α and authentic DM α expressed in Raji cells were subjected to Western blot hybridization.

The antiserum obtained from the mice immunized with the recombinant DM α expressed in E. coli, well recognized the recombinant DM α expressed in E. coli, but not the authentic DM α expressed in Raji cells. In contrast, the antiserum taken from the mice immunized with the DM α expressed in the baculovirus system, well responded to the recombinant DM α as well as the authentic DM α expressed in Raji cells. This result suggests that the recombinant DM α expressed in E. coli is likely to be different from the normal DM α proteins in antigenicity. It is believed

that this difference might cause the previous art to fail in producing the monoclonal antibody which is able to react with the normal DM α, when mice were immunized with the DM α expressed in E. coli (Kim et al., Mol. Cells, 6, 684, 1996).

INDUSTRIAL APPLICABILITY

As described hereinbefore, the monoclonal antibody 3-6-A of the present invention shows a potent reactivity to the authentic DM α normally expressed in DCs and Raji cells. Particularly noteworthy is that the monoclonal antibody 3-6-A is very specific and strong binding capacity to DCs among the PBL. The monoclonal antibody 3-6-A stains DC on their surface as well as in the cytoplasm. Being very helpful in studying the functions of DM molecules, therefore, the monoclonal antibody 3-6-A of the present invention can be used as a DC-specific surface marker, which seems to be strong enough to allow positive selection of DCs from PBLs. The present invention will be very useful in the biomedical industry, contributing to the advance in the research on the immunotherapy against cancers or other chronic diseases.

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT
OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO: Bae, Yong- Soo
Expo apt. 308-704, Jeonmin-dong, Yusong-ku, Taejon 305-390,
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
Hybridoma cells : KHB-DM	**KCTC 0485BP**

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:
[x] a scientific description
[ ] a proposed taxonomic designation
(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on **May 29 1998.**

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: Korea Research Institute of Bioscience and Biotechnology Korean Collection for Type Cultures	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):
Address: KCTC, KRIBB #52, Oun-dong, Yusóng-ku, Taejón 305-333, Republic of Korea	Bae K. S. Kyung Sook Bae, Curator Date: **June 05 1998**

CLAIMS

1. A monoclonal antibody, 3-6-A, produced in a hybridoma cell, KHB-DM (KCTC-0485BP), which is highly specific to the surface of dendritic cells among peripheral blood leukocytes.

2. A hybridoma cell, KHB-DM, with a Deposition No. of KCTC-0485BP, which produces the monoclonal antibody 3-6-A of claim 1.

3. A recombinant plasmid pBlueBacHis2A-DMα, comprising a 609 bp DNA fragment coding for an extracellular region of a DMα gene, said extracellular region corresponding to a base sequence covering between 122 and 732.

4. A recombinant baculovirus, Bac-N-Blue-DMα, prepared by co-transfecting the recombinant plasmid, pBlueBacHis2A-DMα, of claim 3 and a baculovirus full-length DNA, Bac-N-Blue, into High-5 cells.

5. A method for probing dendritic cells from a sample, comprising the steps of:

binding an antibody to a DMα protein expressed on the surface of the dendritic cells in the sample, said antibody specifically reacting only with the

dendritic cells among the peripheral blood leukocytes, said sample containing or being expected to contain the dendritic cells; and

marking the antibody bound to the DM α expressed on the surface of the dendritic cells to detect the cells stained with the antibody.

6. A method as set forth in claim 5, wherein said antibody is the monoclonal antibody, 3-6-A, of claim 1.

7. A method as set forth in claim 5, wherein said marking step is conducted by staining the antibody-attached DM α protein or the antibody-coated cells with a second antibody conjugated with fluorescence iso-thiocyanate (FITC) and visualizing the cells or DM α protein in any association with dendritic cells.

FIG. 1

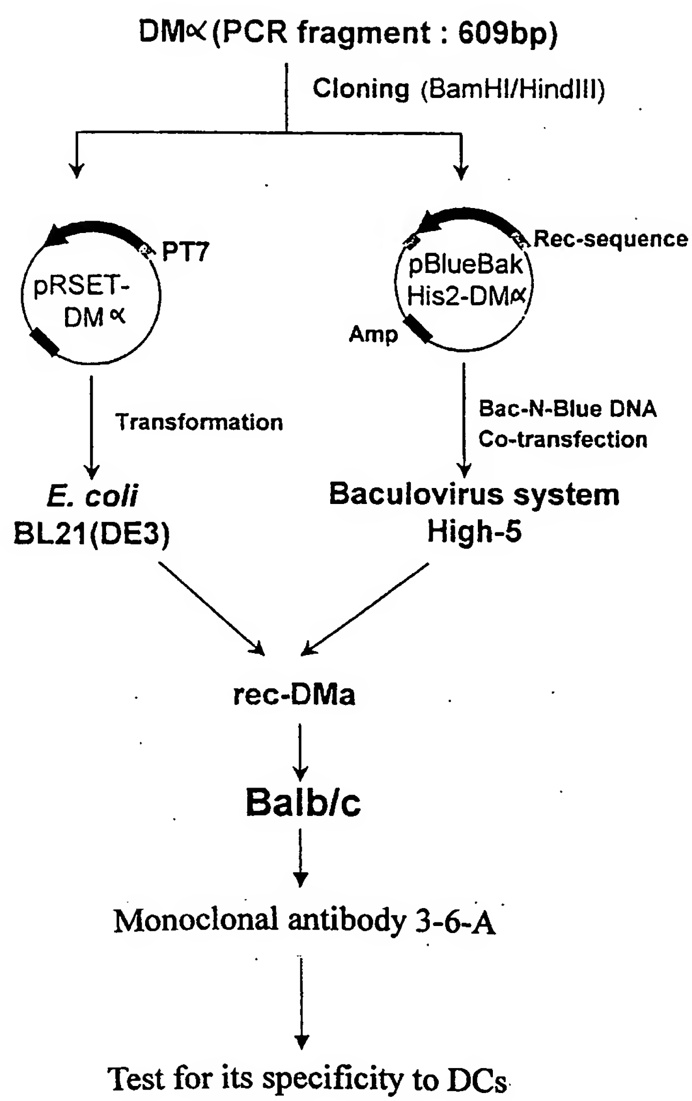

DMα (PCR fragment : 609bp)
Cloning (BamHI/HindIII)
PT7
pRSET-DMα
Rec-sequence
pBlueBak His2-DMα
Amp
Transformation
Bac-N-Blue DNA Co-transfection
E. coli BL21(DE3)
Baculovirus system High-5
rec-DMa
Balb/c
Monoclonal antibody 3-6-A
Test for its specificity to DCs

FIG. 2

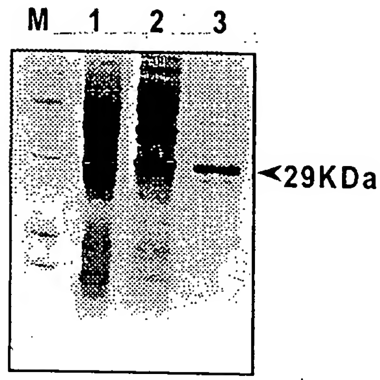
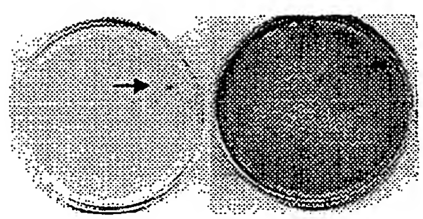


FIG. 3



(A) (B)

FIG. 4

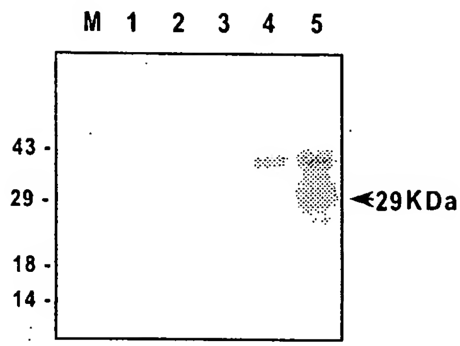


FIG. 5

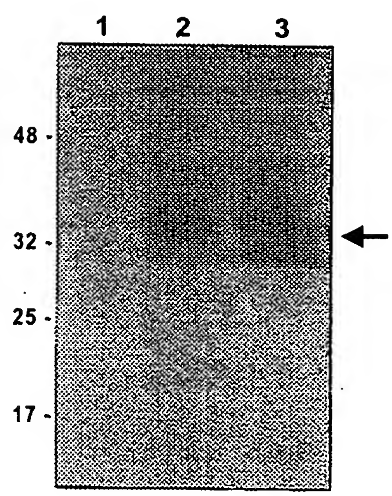


FIG. 6

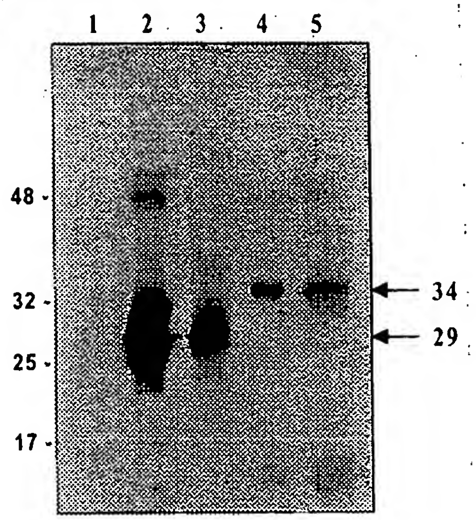


FIG. 7

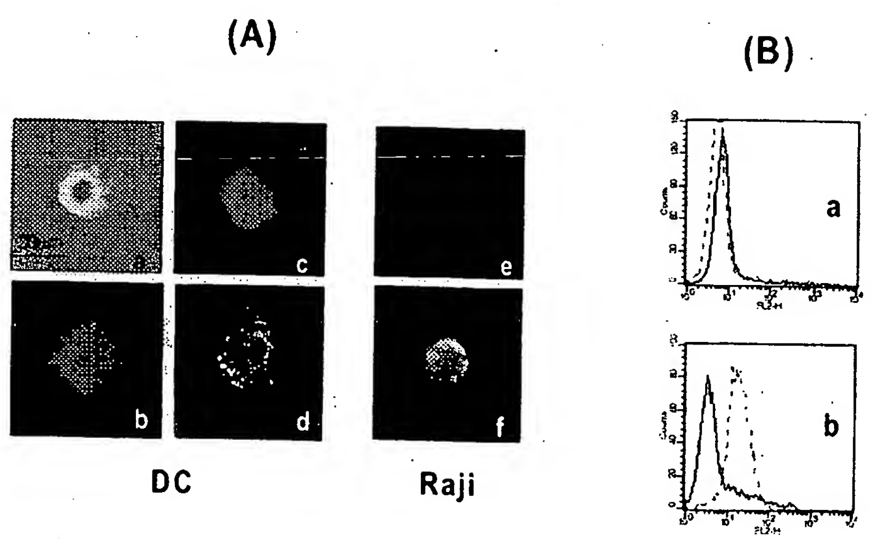


FIG. 8

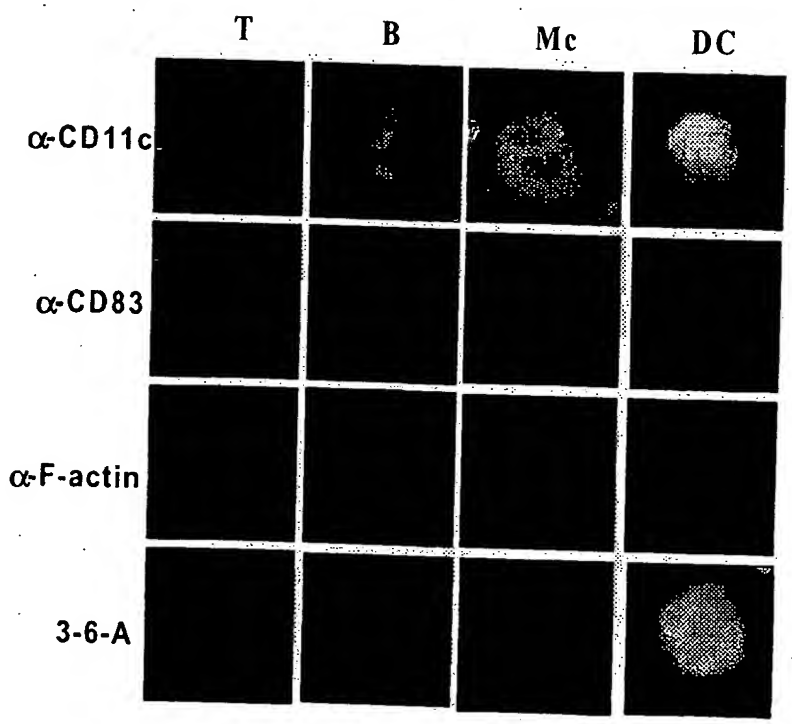


FIG. 9

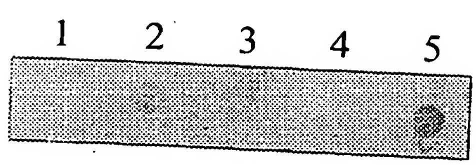
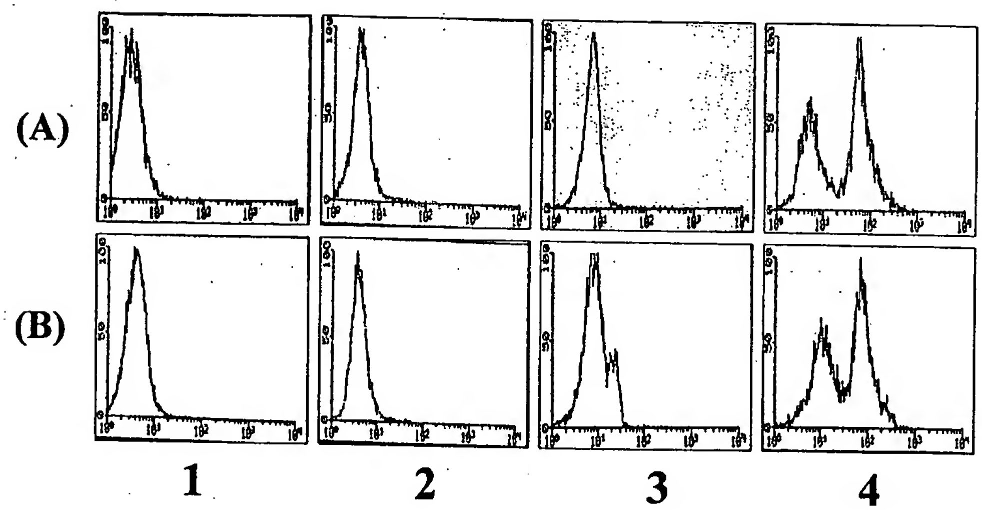


FIG. 10



INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 99/00212

A. CLASSIFICATION OF SUBJECT MATTER

IPC[6]: C 12 N 5/20, 15/12; C 07 K 16/30; C 12 N 15/70, 15/85

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[6]: C 12 N 5/20, 15/12; C 07 K 16/30; C 12 N 15/70, 15/85

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, EPODOC, CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 93/04 187 A1 (MEDICAL RESEARCH COUNCIL), 04 March 1993 (04.03.93), page 3, lines 8-21; page 9, line 16 - page 10, line 15; page 20, line 4 - page 21, line 6; claims 1-7, 9.	1,2,5,7
A	WO 95/12 404 A1 (CANTERBURY HEALTH LIMITED), 11 May 1995 (11.05.95), claims 1,6,8,9,13,15-21,24.	1,2,5,7
A	WO 95/15 340 A1 (THE BOARD OF TRUSTEES OF LELAND STANFORD JUNIOR UNIVERSITY), 08 June 1995 (08.06.95), claims 1,4,5,7-9.	1
A	WO 97/48 798 A1 (GENETICS INSTITUTE, INC.), 24 December 1997 (24.12.97), pages 1,2; page 7, line 29 - page 8, line 27.	3

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
„A" document defining the general state of the art which is not considered to be of particular relevance
„E" earlier application or patent but published on or after the international filing date
„L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
„O" document referring to an oral disclosure, use, exhibition or other means
„P" document published prior to the international filing date but later than the priority date claimed

„T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
„X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
„Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
„&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 August 1999 (13.08.99)	24 August 1999 (24.08.99)

Name and mailing adress of the ISA/AT	Authorized officer
Austrian Patent Office Kohlmarkt 8-10; A-1014 Vienna Facsimile No. 1/53424/200	Mosser Telephone No. 1/53424/437

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/KR 99/00212

Im Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche		Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets		Datum der Veröffentlichung Publication date Date de publication
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			GB A0	9117522	02-10-1991
WO A1	9512404	11-05-1995	AU A1	10872/95	23-05-1995
			US A	5806529	15-09-1998
WO A1	9515340	08-06-1995	keine - none - rien		
WO A1	9748798	24-12-1997	AU A1	33121/97	07-01-1998
			AU A1	33986/97	07-01-1998
			AU A1	33987/97	07-01-1998
			AU A1	34914/97	07-01-1998
			CA AA	2257827	24-12-1997
			CA AA	2257834	24-12-1997
			CA AA	2257842	24-12-1997
			CA AA	2257847	24-12-1997
			EP A1	910637	28-04-1999
			EP A1	914429	12-05-1999
			EP A2	914430	12-05-1999
			EP A2	920499	09-06-1999
			US A	5807703	15-09-1998
			WO A1	9748800	24-12-1997
			WO A2	9748799	24-12-1997
			WO A2	9748801	24-12-1997
			WO A3	9748799	19-02-1998
			WO A3	9748801	14-05-1998
			US A	5807709	15-09-1998
			US A	5827688	27-10-1998
			US A	5837490	17-11-1998